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PRINCIPAL INVESTIGATOR: Luis Ulloa, Ph.D.

CONTRACTING ORGANIZATION: University of Medicine and Dentistry of New Jersey  
Newark, NJ 07101

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14. ABSTRACT Many promising strategies in experimental models of hemorrhage have failed in clinical trials, in part because classical experimental models may not mimic clinical settings. Unlike classical experimental models, hemorrhage in critical care is normally associated with collateral trauma that affects the physiological responses during resuscitation. Unlike rodents, swine are an optimal species donor for experimental hemorrhage as they have an anatomy, physiology and hemodynamic responses that closely resembles human. Here, we analyze whether ethyl pyruvate can provide a therapeutic anti-inflammatory value to resuscitation fluids in porcine hemorrhage with trauma. Ethyl pyruvate prevented systemic TNF levels, hyperglycemia, aspartate aminotransferase and preserved the intrinsic coagulation pathway. Resuscitation with ethyl pyruvate attenuated TNF levels in the spleen, liver and intestine. The most significant effects were found in the terminal ileum were ethyl pyruvate inhibited TNF levels, restrained myeloperoxidase activity, preserved the intestinal epithelium, and prevented the appearance of bacterial endotoxin in the serum. Unlike observed in rodents, ethyl pyruvate did not attenuate TNF levels in the lung and the heart, providing a potential explanation for its failure in clinical trials of cardiopulmonary bypass. These results suggest that ethyl pyruvate provided significant effects in porcine hemorrhage previously undetected in rodents. These results suggest that anti-inflammatory adjuvant in resuscitation fluids can prevent organ damage and it may decrease the susceptibility to secondary sepsis during resuscitation.					
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## Introduction

Hemorrhage is a leading cause of death, despite the recent advances in resuscitation and critical care [1, 2]. After losing over 50% of the total blood volume, the system is unable to reestablish tissue perfusion and the animals succumb to lethal organ dysfunction. Conventional resuscitation fluids are designed to restore circulatory volume and tissue perfusion, but they failed to prevent the inflammatory responses [3, 4]. Controlled production of inflammatory cytokines such as tumor necrosis factor (TNF) can be beneficial to confine infection or trauma by inducing localized blood clotting. However, excessive TNF production causes necrosis, organ failure and cardiovascular shock than can be more dangerous than the original hemorrhagic insult [5, 6]. Recombinant TNF can trigger a variety of hemodynamic symptoms similar to that found in hemorrhagic shock. Conversely, TNF neutralization can prevent cardiovascular shock and improve organ function in some experimental models of hemorrhage [7-10]. Thus, anti-inflammatory strategies to restrain TNF production can provide a therapeutic potential during resuscitation.

Conventional resuscitation fluids such as Ringer's lactate are basically lactate-supplemented saline solutions that resemble plasma composition [11, 12]. Advanced resuscitation fluids include Hextend, a colloidal plasma volume expander containing 6% hydroxyethyl starch in Ringer's lactate [13]. The hetastarch in Hextend creates oncotic pressure, normally provided by blood proteins that permit retention of intravascular fluid. Resuscitation with Hextend required less intravenous fluid, attenuated organ injury, and provided a significant survival benefit over saline in hypotensive resuscitation [14-16]. Lactate does not provide any therapeutic value, and may even exacerbate lactic acidosis during hypovolemia [17]. Unlike lactate, pyruvate, a structurally similar compound, is a potential antioxidant and anti-inflammatory that protects mammalian cells from cytotoxicity [18]. Pyruvate appears to be beneficial in experimental models of stroke and hemorrhage [19]. Despite its therapeutic potential, the clinical use of pyruvate is hampered by its instability and toxicity [18, 20]. Aqueous solutions of pyruvate degrade via condensation and cyclization reactions which interfere with the tricarboxylic acid cycle, inhibiting mitochondrial respiration [18, 21]. These limitations appear to be avoided by ethyl pyruvate, a more stable lipophilic pyruvate derivative [22, 23]. Ethyl pyruvate is a stable, soluble, non-toxic compound that lacks the potential toxicity of pyruvate and can provide a similar anti-inflammatory potential. Ethyl pyruvate is classified as GRAS (generally regarded as safe) by the FDA and it has been recently used in clinical trials.

Recent studies indicate that ethyl pyruvate inhibits TNF production in human and murine macrophages [23, 24]. *In vivo*, ethyl pyruvate attenuated systemic inflammation and improved survival in experimental sepsis [23-28] and hemorrhage [29-32] using rodents. However, a recent study indicates that a single dose of ethyl pyruvate may worsen survival in endotoxemia [33] depending on the dose and time of administration [34]. These results reveal the need to study the factors affecting the mechanism of action of ethyl pyruvate [34]. These studies are of significant interest since a recent clinical trial failed to prove a therapeutic potential of ethyl pyruvate in cardiopulmonary bypass [35]. Indeed, many promising strategies in experimental models of hemorrhage have provided limited benefits in clinical trials, in part because classical experimental hemorrhage is performed in rodents and lacks the collateral trauma that exacerbate the inflammatory and physiological responses during hemorrhage and resuscitation [1, 2]. Unlike rodents, swine have cardiovascular, gastrointestinal, and physiological responses that closely resemble that found in humans [36-39]. We analyzed the therapeutic potential of ethyl pyruvate during resuscitation in rodents and porcine models of experimental hemorrhage. Blood and organs were analyzed to determine the characteristic markers of organ function and the inflammatory responses and potential survival benefits. Our results suggest that ethyl pyruvate may provide a therapeutic anti-inflammatory potential to resuscitation fluids.

Here we have analyzed whether ethyl pyruvate can provide an anti-inflammatory therapeutic potential to Hextend solution to improve survival in experimental models of :

- I. Lethal hemorrhage resuscitation with limited volume.
- II. Awake hemorrhage in rats without anesthesia.
- III. Hemorrhage with trauma induced by broken femur and soft tissue injury.
- IV. Porcine hemorrhage with trauma and soft tissue injury.

The experimental design described five experimental groups:

- (1) Hemorrhage with NO resuscitation with 90-100% mortality at 6 hours.
- (2) Hemorrhage and resuscitation with Hextend resulting in 30-50 % mortality at 6 hours.
- (3) Hemorrhage and resuscitation with HxEP50mM (Hextend containing 50mM ethyl pyruvate).
- (4) Hemorrhage and resuscitation with HxEP25mM (Hextend containing 25mM ethyl pyruvate).
- (5) Hemorrhage and resuscitation with HxEP5mM (Hextend containing 5mM ethyl pyruvate).

## **Body of Results**

***Ethyl pyruvate provides therapeutic potential to resuscitation fluids in rodents with hemorrhage with trauma.*** Hemorrhagic shock required withdrawing  $18.2 \pm 3.6$  mL blood/Kg body weight and the maintenance of that blood pressure for another 15 minutes required another  $6 \pm 2.5$  mL blood/Kg body weight. Animals without resuscitation (NR) did not reestablish normal blood pressure (Fig. 1A), and died within the first five hours after hemorrhage (Fig. 1B). Resuscitation with Hextend (HXT) allowed the animals to recover normal blood pressure and reestablish tissue perfusion, but still over 60% of the animals died within the first five hours after hemorrhagic shock. Resuscitation with Hextend protected 40% of the animals in a statistical significant manner ( $n=9$ ;  $P<0.001$ , Logrank Test; HXT vs control). The addition of 50 mM ethyl pyruvate to Hextend solution provided a therapeutic potential to rescue all the animals from lethal hemorrhage ( $P<0.05$ , Logrank Test; HEP vs HXT). All the animals resuscitated with Hextend supplemented with 50 mM ethyl pyruvate survived ( $n=7$ /group). Ethyl pyruvate improved survival in a concentration dependent manner. The addition of 25 mM but not 5 mM ethyl pyruvate to Hextend induced a statistical significant protection ( $P=0.01$  and  $P=0.6$  respectively, Logrank Test) as compared with Hextend solution. Addition of 25 mM ethyl pyruvate to Hextend protected 80% of the animals with hemorrhagic shock, whereas 5 mM ethyl pyruvate protected 50% of the animals ( $n=7$ /group). By design, our analyses excluded those animals that died during the surgical procedure and counted only those animals that complete the resuscitation treatment. All nonsurvival animals died within the first five hours after hemorrhagic shock. All the animals that passed this critical period, survived the hemorrhagic shock, and no late deaths were found although the animals were followed for up to seven days, suggesting that ethyl pyruvate induced a lasting protection and it did not merely delay the pathologic onset.

***Ethyl pyruvate prevents systemic inflammatory responses during hemorrhage in rodents with hemorrhage with trauma.*** The animals were subjected to closed femoral fracture prior the hemorrhage to mimic collateral trauma during critical care. Tumor necrosis factor (TNF) is a characteristic inflammatory cytokine that acts a cardiodepressant factor in resuscitation contributing to cardiovascular shock. TNF concentration was measured in the serum and organs TNF levels were normalized with protein concentration. Animals subjected to hemorrhage without trauma have lower serum TNF levels as compared to those animals with hemorrhage and trauma (Hemorrhage(H) =  $157.5 \pm 27.02$  pg/serum mL vs Hemorrhage with trauma (TH) =  $277.1 \pm 72.79$  pg/serum mL;  $p<0.05$ ; Fig. 3). Among the major organs, TNF concentration was higher in the heart and the spleen in both hemorrhage and hemorrhage with trauma. The higher systemic TNF levels found during hemorrhage with trauma associated with significantly higher levels of TNF in the heart, the spleen and the liver but not in the lung. These results indicated that trauma enhances systemic inflammatory responses during hemorrhage leading to higher concentration of pro-inflammatory and cardiodepressant factors in the heart.

## **Porcine Hemorrhage**

***Experimental Model Development and Characterization.*** First, we developed a lethal model of hemorrhage in swine similar to that used to study ethyl pyruvate in rodents[40]. Rodents were bled for 15 min to reach a mean arterial blood pressure of 35-40 mmHg, and that blood pressure was maintained for another 15 min[41]. All non-treated rodents subjected to this protocol died within the first five hours after

hemorrhage[41]. Unlike rodents, all swine without resuscitation survived this procedure. Then, we analyzed the conditions to achieve a similar mortality pattern as that described in rodents. Our experimental strategy was to bleed the jugular vein for 15 min to reach a mean arterial blood pressure of 20-25 mmHg and maintenance of that blood pressure for another 30 min. Similar to rodents, all swine without resuscitation died within the first seven hours after hemorrhage. However, all the swine that received resuscitation (15 mL/Kg body weight) with Hextend alone, or supplemented with 50mM ethyl pyruvate, survived the next seven hours after resuscitation. Characteristic pathological markers of hemorrhage were assessed by blood chemistry at three hours after hemorrhage, as described in previous studies [40]. Animals without resuscitation were characterized by uremia, metabolic acidosis and hyperglycemia. Both resuscitation with Hextend, and ethyl pyruvate failed to prevent uremia. Animals without resuscitation had a characteristic acidic blood pH, increased anion gap (AnGap) and negative base excess of extracellular fluid (BEecf). Resuscitation with Hextend alone or with ethyl pyruvate improved metabolic acidosis, anion gap and BEecf. These effects on metabolic acidosis did not correlate with changes in bicarbonate, gases (total and partial CO<sub>2</sub>), or characteristic electrolytes (sodium, chloride). Neither hemorrhage nor resuscitation with Hextend or ethyl pyruvate affected serum bicarbonate levels in any of the experimental groups. One of the most significant effects of hemorrhage and the therapeutic potential of ethyl pyruvate was found in hyperglycemia. Hemorrhage induced a characteristic hyperglycemic response ( $P < 0.05$ , NR vs. Control). Resuscitation with Hextend did not prevent hyperglycemia. But, resuscitation with ethyl pyruvate significantly decreased glucose levels toward normal values. The only statistically significant difference between the resuscitation treatments was the potential of ethyl pyruvate to significantly prevent hyperglycemia as compared with those animals resuscitated with Hextend alone ( $P < 0.01$ , HEP vs. HXT).

***Ethyl Pyruvate attenuated blood clotting time.*** The effects of ethyl pyruvate in both the contact activation (intrinsic) and the tissue factor (extrinsic) coagulation pathway were analyzed using the celite (cACT), and the kaolin (kACT) activated clotting time, the prothrombin time (PT), and the international normalized ratio (INR) (Fig 2). Both intrinsic activity tests measure thrombin activation of coagulation cascade, but have slightly different anti-coagulant ranges and sensitivities to protease inhibitors. In our experimental model, hemorrhage induced a more significant activation of the intrinsic coagulation pathway as determined by the activated clotting time than the extrinsic pathway. Hemorrhage significantly increased both the celite and kaolin clotting time in a similar fashion ( $P < 0.05$ , NR vs. Control). However, celite-ACT tests provided more reproducible data with lower standard deviation giving more statistically significant results. Resuscitation with Hextend failed to significantly prevent this process, and these animals had activated clotting times significantly higher than that in control animals ( $P < 0.05$ , HXT vs. NR). In contrast, the blood from the animals resuscitated with ethyl pyruvate had clotting times significantly lower than the animals treated with Hextend ( $P < 0.05$ , HEP vs. HXT), and statistically similar to that in control animals ( $P > 0.05$ , HEP vs. Control). The extrinsic clotting tendency was measured by prothrombin clotting time (PT) and the international normalized ratio (INR). Unlike previous tests, all groups have a statistically similar prothrombin clotting time and international normalized ratio, as they were not significantly affected by either hemorrhage or resuscitation with Hextend or ethyl pyruvate.

***Ethyl pyruvate provided anti-inflammatory potential and prevented organ damage during resuscitation.*** Previous studies indicated that serum TNF peaks at approximately 2-3 hours after resuscitation [42]. We analyzed TNF levels in the serum and the major organs of the swine at three hours after hemorrhage. Control animals have significant serum TNF levels induced by the trauma and the sham hemorrhagic surgery (Fig. 3A). Hemorrhagic animals without resuscitation had over 2-fold higher serum TNF levels than the control animals ( $P < 0.05$ , NR vs. Control,  $n=4$ /group). Resuscitation with Hextend alone failed to attenuate serum TNF levels in hemorrhagic animals. However, resuscitation with Hextend containing 50mM ethyl pyruvate (HEP) significantly decreased serum TNF levels by ~60% as compared to Hextend alone ( $P < 0.05$ , HEP vs. HXT,  $n=4$ /group). Serum TNF levels in the animals resuscitated with ethyl pyruvate were statistically similar to those in the control animals ( $P > 0.05$ , HEP vs. Control). Hemorrhage did not significantly increase TNF levels in the lung or the heart as compared to that in the sham animals (Fig. 3B, C). Hemorrhage increased TNF levels in the spleen and liver by nearly 2-fold, a pattern similar to that found in the serum (Fig. 3D, E). The largest induction of TNF was found in the terminal ileum, where hemorrhage increased TNF levels by over 2.5-fold (Fig. 3F,  $P < 0.05$ , NR vs. Control, ANOVA). Resuscitation with Hextend failed to inhibit TNF levels in any organ. In contrast, ethyl pyruvate significantly

inhibited TNF levels in the major organs including the spleen, liver and terminal ileum toward values statistically similar to those in the control animals. Similar results were found, when TNF RNA levels were analyzed by real-time PCR. As seen for protein, the largest increase of TNF RNA levels induced by hemorrhage was found in the terminal ileum with over 2-fold induction, followed by the spleen and liver (data not shown). Resuscitation with Hextend supplemented with ethyl pyruvate was particularly efficient reducing TNF RNA levels in the terminal ileum toward levels statistically similar to that found in control sham animals. Organ damage was analyzed by measuring aspartate aminotransferase (AST) and myeloperoxidase (MPO) activities. Hemorrhage significantly increased aspartate aminotransferase activities in the serum. Unlike Hextend, ethyl pyruvate statistically prevented serum aspartate aminotransferase activity ( $P<0.05$ , HEP vs. HXT, Fig 4A), toward values statistically similar to those in control animals ( $P>0.05$ , HEP vs. Control). Hemorrhage significantly increased myeloperoxidase activity, a characteristic measure of tissue neutrophil infiltration and inflammation, in all the organs ( $P<0.05$ , NR vs. Control; Fig 4B-F). The most significant induction of myeloperoxidase was found in the heart, liver and terminal ileum ( $P<0.01$ , NR vs. Control). Resuscitation with Hextend alone failed to prevent myeloperoxidase levels in any organ as compared to those animals without resuscitation. Resuscitation with ethyl pyruvate prevented myeloperoxidase levels in the lung, heart and gut but not in the spleen and liver. The most significant effects were found in the terminal ileum, where ethyl pyruvate inhibited myeloperoxidase levels by 40% as compared to those animals resuscitated with Hextend.

#### ***Ethyl pyruvate preserved intestinal epithelium, and prevented bacterial leakage.***

Sections from swine terminal ileum were collected at three hours after hemorrhage and stained for Hematoxylin-Eosin histology (Fig 5). The intact lamina propria of control (C) sham animals showed normal intestinal morphology with the characteristic epithelial distribution (Grade 0). Animals without resuscitation (NR) had denuded villi with increased cellularity, dilated capillaries, and lamina propria exposed (Grade 4). Resuscitation with Hextend alone (HXT) induced massive epithelial lifting down the side of the villi and a few tips denuded (Grade 3), diminution of villous height and gut mucosal goblet cell number. Resuscitation with Hextend supplemented with ethyl pyruvate (HEP) showed capillary congestion, development of subepithelial Gruenhagen's space, usually at the apex of some villi (Grade 1), normal villous height and mucosal goblet cell number. These results suggest that resuscitation with ethyl pyruvate can preserve intestinal epithelium and prevent bacterial leakage. Bacterial endotoxin was measured in the blood by using the Limulus Amebocyte Lysate (LAL) test (Fig 5E). Hemorrhage disrupted intestinal morphology, which correlated with a significant increase of bacterial endotoxin in the blood ( $P<0.01$  NR vs. Control, ANOVA). Resuscitation with Hextend did not prevent these levels of serum LPS ( $P<0.01$ , HXT vs. Control). However, resuscitation with ethyl pyruvate preserved intestinal morphology and significantly prevented serum LPS levels as compared with those animals resuscitated with Hextend ( $P<0.05$ , HEP vs. HXT). Animals resuscitated with ethyl pyruvate had serum bacterial endotoxin levels statistically similar to that found in control animals. The serum bacterial endotoxin levels for all samples correlated with the integrity of the intestinal epithelium.

## **Key Research Accomplishments**

Our results indicate that ethyl pyruvate can provide a therapeutic anti-inflammatory value to advanced resuscitation fluids such as Hextend. This therapeutic potential was translated in a survival benefits in rodents subjected to different experimental models including: (1) experimental hemorrhage and resuscitation with limited volume; (2) Awake hemorrhage in rats without anesthesia, (3) Hemorrhage with trauma induced by broken femur and soft tissue injury. These survival benefits were associated with an anti-inflammatory potential of the ethyl pyruvate to prevent systemic inflammation and TNF production during resuscitation.

In porcine hemorrhage ethyl Pyruvate attenuated blood clotting time in porcine hemorrhage. Ethyl pyruvate also provided anti-inflammatory potential and prevented organ damage during resuscitation in porcine hemorrhage. In contrast to Hextend, ethyl pyruvate significantly inhibited TNF levels in the major organs including the spleen, liver and terminal ileum toward values statistically similar to those in the control animals. Ethyl pyruvate preserved intestinal epithelium, and prevented bacterial leakage.

## Reportable Outcomes

The results of this research have been published in the following articles:

Cai B, Chen F, Xingchun L, Miller E, Szabo C, Deitch E, Ulloa L. "Anti-inflammatory adjuvant in resuscitation fluids improves survival in hemorrhage" *Crit Car Med* 2009, 37(3) 860-8.

Cai B, Brunner M, Wang H, Wang P, Deitch EA, Ulloa L. "Ethyl pyruvate improves survival in awake hemorrhage" *J. Mol Med* 2009 Jan 28. [Epub ahead of print].

Cai B, Deitch EA, Grande D, Ulloa L. "Anti-inflammatory resuscitation improves survival in hemorrhage with trauma." *J Trauma* (In press).

Dong W, Cai B, Peña G, Pisarenko V, Doucet D, Lee M, Sharpe S, Lu Q, Xu D, Brunner M, Ramos L, Deitch E & L Ulloa. "Anti-inflammatory Resuscitation Prevents Organ Damage In Porcine Hemorrhage" (Submitted for publication)

## Conclusion

Our results suggest that ethyl pyruvate can provide a significant therapeutic anti-inflammatory potential to Hextend. These results are significant because resuscitation with Hextend supplemented with ethyl pyruvate improves survival in different experimental models of hemorrhage. The most significant results correlating with the therapeutic potential of ethyl pyruvate during resuscitation were its anti-inflammatory potential to inhibit systemic inflammation similar as described in other studies including experimental sepsis [23, 24, 31]. Our results are particularly significant in four considerations. First, this is an experimental model of severe hemorrhage with over 75% of estimated blood volume lost. Second, shed blood was considered lost and it was not reinfused. Moreover, animals were treated with a small volume of 15mL/Kg resuscitation (equivalent to 1000mL of Hextend in a 70 Kg patient) that represents approximately 50% of the total shed blood volume. Third, our studies use Hextend as control solution. This is a critical consideration as recent studies indicate that resuscitation with Hextend prevents multiple organ injury [43] and improves short-time survival as compared with saline [14]. Indeed, all our animals resuscitated with control solution survived the initial response (<4 hrs) but 90% of them died in the secondary inflammatory phase 4-10 hours post-resuscitation. Fourth, the animals were followed for up to one week to analyze total survival including late deaths. Together, these considerations are of particular interest in scenarios of limited supplies including critical care of both military operations and civil mass casualties. These considerations are particularly pronounced on the battlefield characterized by low supplies of resuscitation fluid, long transport times to a medical facility and hemorrhage associated with an exacerbated inflammatory response produced by collateral trauma. Resuscitation with Hextend supplemented with ethyl pyruvate induced a complete and lasting protection and survival in experimental models of hemorrhage including lethal hemorrhage with limited resuscitation, hemorrhage with trauma, and awake hemorrhage.

Unlike resuscitation with Hextend, ethyl pyruvate attenuated TNF production in the spleen, liver and the terminal ileum, prevented systemic inflammation, organ damage and bacterial leakage in porcine hemorrhage. One of the most significant effects of ethyl pyruvate was its potential to prevent systemic TNF levels and organ injury, yet these two processes had a different pattern. Ethyl pyruvate inhibited TNF levels in the serum, spleen, liver and terminal ileum, yet it did not prevent myeloperoxidase activity in the spleen and the liver. In contrast, ethyl pyruvate did not inhibit TNF response in the lung and heart, and yet it prevented myeloperoxidase activity in those organs. It should be considered that the inability of ethyl pyruvate to control TNF levels in lung and heart may have prevented therapeutic potential of ethyl pyruvate in the recent clinical trials of cardiopulmonary bypass. Ethyl pyruvate may not have provided a therapeutic



potential during the reperfusion of the lung/heart [44]. Unlike rodents, our studies in porcine hemorrhage did not show short-term survival benefits of ethyl pyruvate as compared with Hextend. According to IACUC recommendations, our studies were limited to non-survival surgery where the swine must be kept anesthetized and continuously monitored. Swine were observed for up to seven hours after surgery. All animals resuscitated with Hextend alone or Hextend supplemented with ethyl pyruvate survived this time period. Since evacuation time in military operations can be significantly higher (>14hrs), alternative strategies and future studies with longer periods of observation will be needed to determine a potential survival benefit for ethyl pyruvate.

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## **Figure Legends.**

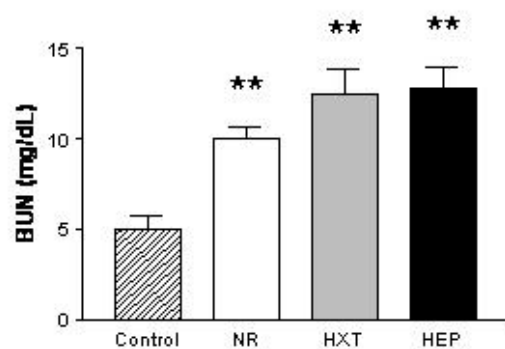
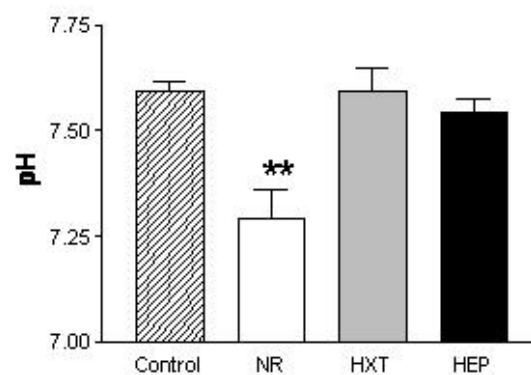
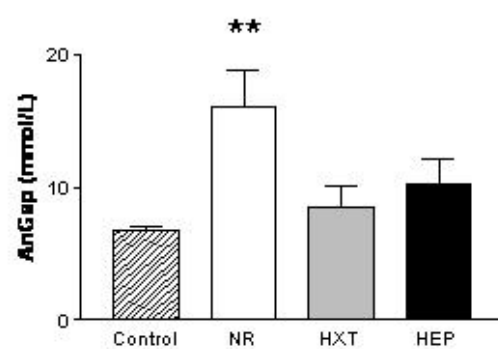
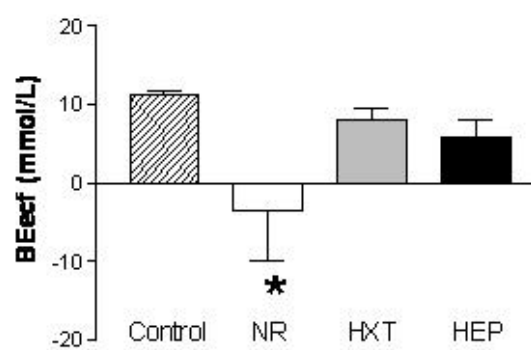
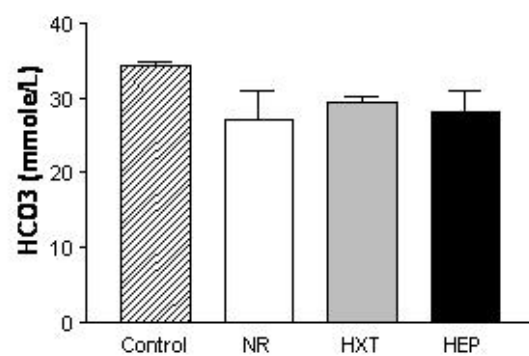
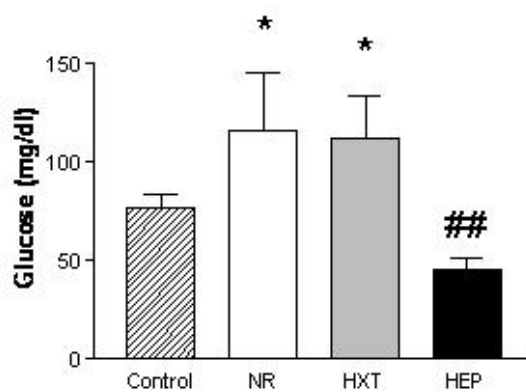
**Figure 1 Blood chemistry analyses during resuscitation.** Blood from control adult male Yorkshire swine, or hemorrhagic animals without resuscitation (NR), or resuscitated with 15mL/Kg (i.v.) Hextend (HXT) or Hextend supplemented with 50 mM ethyl pyruvate (HEP) was collected three hours after hemorrhage to analyze (A) Blood urea nitrogen (BUN), (B) pH, (C) the anion gap, (AnGap), (D) base excess of extracellular fluid (BEecf), (E) bicarbonate ( $\text{HCO}_3$ ) and (F) Glucose. \* represents  $P < 0.05$  vs. Control. \*\* represents  $P < 0.01$  vs. Control. # represents  $P < 0.05$ , HEP vs. HXT. ## represents  $P < 0.01$ , HEP vs. HXT. n = 4 animals/group.

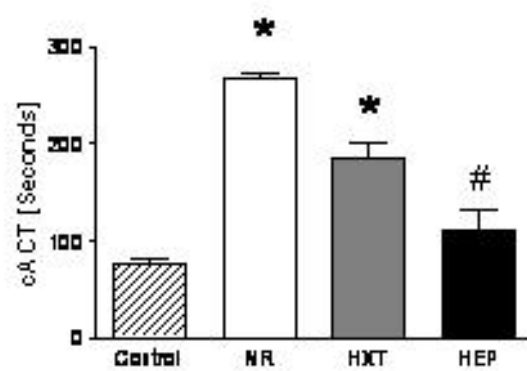
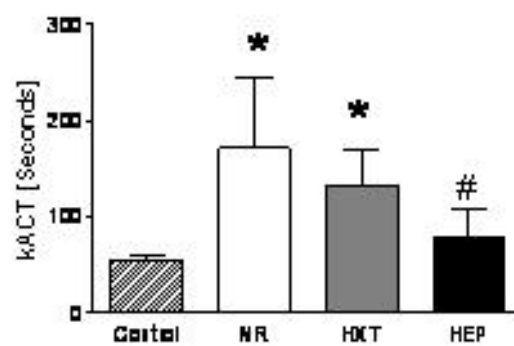
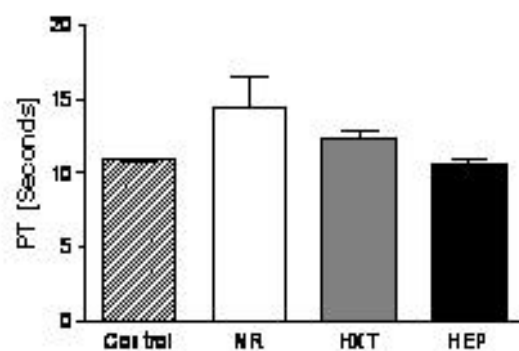
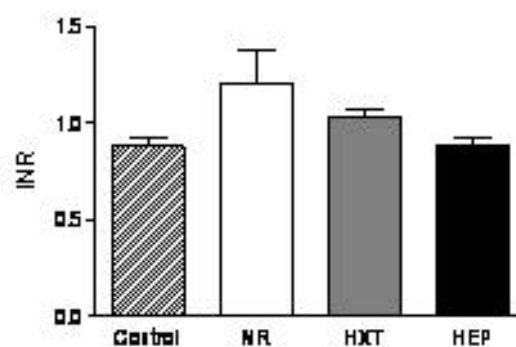
**Figure 2. Ethyl pyruvate decreased the intrinsic coagulation pathway.** Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or treated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP). Blood coagulation tendency was determined by analyzing the intrinsic: (A) celite Activated Clotting Time (cACT), (B) kaolin Activated Clotting Time (kACT), and the extrinsic coagulation pathway: (C) prothrombin time (PT), and (D) the international normalized ratio (INR). \* represents  $P < 0.05$ , NR or HXT vs. Control. # represents  $P < 0.05$ , HEP vs. HXT. n = 4 animals/group.

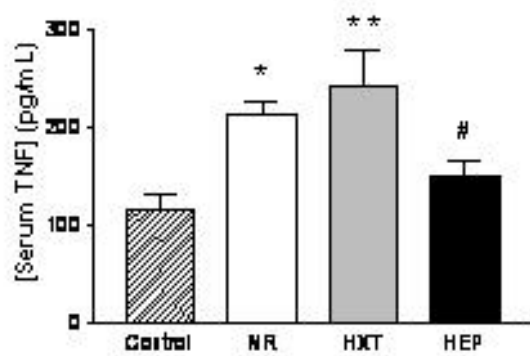
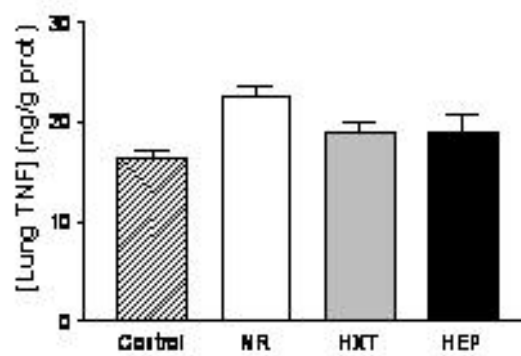
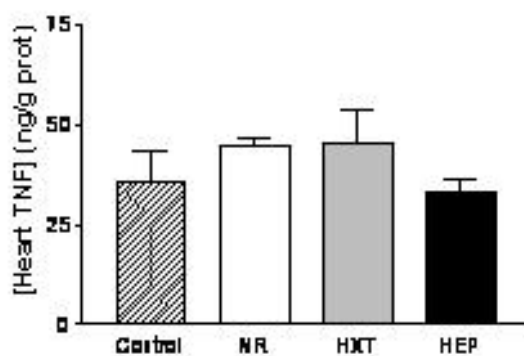
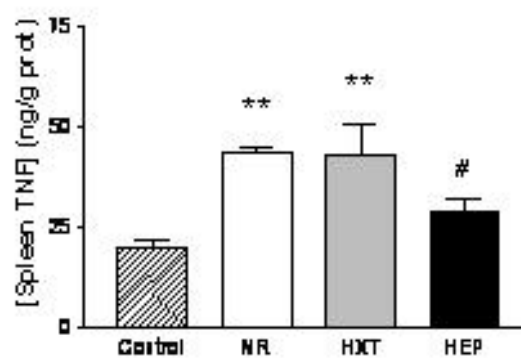
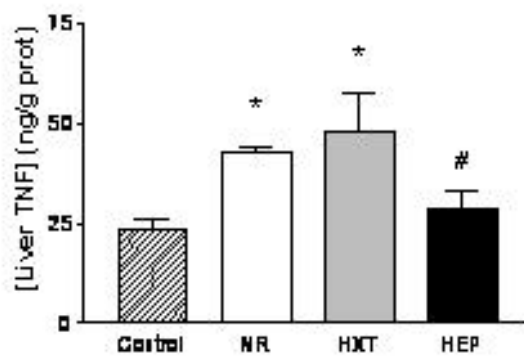
**Figure 3. Resuscitation with ethyl pyruvate provided anti-inflammatory potentials during resuscitation.** Hemorrhagic or control adult male Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or with ethyl pyruvate (HEP). Blood and organs were analyzed 3 hours after hemorrhage to determine TNF concentration in the (A) serum, (B) lung, (C) heart, (D) spleen, (E) liver, and (F) gut. \* represents  $P < 0.05$  vs. Control. \*\* represents  $P < 0.01$  vs. Control. # represents  $P < 0.05$ , HEP vs. HXT. n = 4 animals/group.

**Figure 4. Resuscitation with ethyl pyruvate prevented organ damage in porcine hemorrhage.** Hemorrhagic or control adult male Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP). Serum and organs were analyzed 3 hours after hemorrhage for (A) aspartate aminotransferase (AST) activity in serum, and for myeloperoxidase (MPO) activity in (B) lung, (C) heart, (D) spleen, (E) liver, and (F) Gut. \* represents  $P < 0.05$  vs. Control. \*\* represents  $P < 0.01$  vs. Control. # represents  $P < 0.05$ , HEP vs. HXT. n = 4 animals/group.

**Figure 5. Ethyl pyruvate preserved intestinal epithelium, and prevented serum bacterial endotoxin.** Representative ileum sections were taken for histological examination. Control (C), or hemorrhagic animals without resuscitation (NR), or treated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP) were stained by Hemotoxylin-Eosin. Hemorrhagic animals treated with Hextend showed massive epithelial lifting and denuded villi that was prevented ethyl pyruvate. (E) Bacterial endotoxin (LPS) levels in serum were measured in control (C) or hemorrhagic swine without resuscitation (NR) or treated with Hextend alone (HXT) or supplemented with ethyl pyruvate (HEP). \*\* represents  $P < 0.01$ , NR or HXT vs. Control. # represents  $P < 0.05$ , HEP vs. HXT. n = 4 animals/group.

**A****B****C****D****E****F**

**A****B****C****D**

**A****B****C****D****E****F**